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Principal Investigator: Kristala L. Jones Prather
Assistant Professor, Department of Chemical Engineering

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Abstract/Project Summary

This project was concerned with the development of a methodology for the specification of novel biosynthetic pathways towards organic compounds. Our overall objective is to expand the potential for biological production of small molecules, especially for compounds that have either unknown or intractable natural routes. As a model compound, we chose to design and assemble pathways for the production of glucaric acid, a "top value-added compound from biomass" that has a fully elucidated but very lengthy biological route. As an alternative to the natural pathway, we designed 5 potential routes to glucaric acid and chose two to implement in a recombinant *Escherichia coli* host. During the granting period, we successfully assembled one pathway, the so-called "benchmark pathway," that resulted in the production of glucaric acid at over 1 g/L. Pathway assembly required the isolation of a gene encoding uronate dehydrogenase from the bacterium *Pseudomonas putida*. In order to improve flux through the pathway, we initiated a collaboration with investigators at the University of California, Berkeley, to employ novel enzyme co-localization techniques. A second collaboration was initiated to perform computation-based enzyme engineering for the second pathway. Finally, a publicly-available database of enzymatic transformations (**www.retro-biosynthesis.com**) was created to aid future pathway designs.

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14. ABSTRACT

This project was concerned with the development of a methodology for the specification of novel biosynthetic pathways towards organic compounds. Our overall objective is to expand the potential for biological production of small molecules, especially for compounds that have either unknown or intractable natural routes. As a model compound, we chose to design and assemble pathways for the production of glucaric acid, a top value-added compound from biomass that has a fully elucidated but very lengthy biological route. As an alternative to the natural pathway, we designed 5 potential routes to glucaric acid and chose two to implement in a recombinant Escherichia coli host. During the granting period, we successfully assembled one pathway, the so-called benchmark pathway, that resulted in the production of glucaric acid at over 1 g/L. Pathway assembly required the isolation of a gene encoding uronate dehydrogenase from the bacterium Pseudomonas putida. In order to improve flux through the pathway, we initiated a collaboration with investigators at the University of California, Berkeley, to employ novel enzyme co-localization techniques. A second collaboration was initiated to perform computation-based enzyme engineering for the second pathway. Finally, a publicly-available database of enzymatic transformations (www.retro-biosynthesis.com) was created to aid future pathway designs.

15. SUBJECT TERMS

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I. Scientific and Technical Objectives

Our overall objective is to facilitate the rational design of biosynthetic pathways for the production of unnatural compounds. We are developing tools and attempting to elucidate rules to establish a framework for "retro-biosynthesis," the practice of rationally proposing a synthetic scheme for a target compound from one or more starting substrates, based only on enzyme-mediated transformations. We are especially focused on experimental realization of microbial synthesis, and the limits thereof, in contrast to theoretical predictions. A critical tool is protein engineering for altered substrate specificity, either through rational protein design or random mutagenesis (directed evolution).

We have chosen glucaric acid as a model compound for pathway proposition and assembly in *Escherichia coli*. This compound is naturally-occurring in plants and mammals, but a microbial pathway has not been established. Additionally, the molecule has been declared a "top-ten value added compound from glucose" (Werpy, T. and G. Petersen (2004). "Top value added chemicals from biomass. Volume I: Results of screening for potential candidates from sugars and synthesis gas." National Renewable Energy Lab (NREL) and Pacific Northwest National Lab (PNNL)). The pathway that has been elucidated in mammals is quite complex, consisting of more than 10 reaction steps and an integration with the pentose phosphate pathway. Moreover, the primary product of this natural biosynthetic route is L-ascorbic acid (vitamin C). Thus, we chose to propose rationally-designed pathways for the production of glucaric acid in a microbial host.

Specific objectives are to: (1) propose several pathways for glucaric acid synthesis, (2) select at least one pathway for experimental study, and (3) establish microbial synthesis of glucaric acid. A fourth objective arising from this work is the development of a database for the re-classification of enzyme activities based only on substrate and product functional groups. The database is designed to enable searches more amenable to enzyme selection for biosynthetic pathway proposition.

II. Approach

We propose pathways using an approach that is analogous to that used by organic chemists in proposing synthesis schemes. Key to this approach is the following:

- We consider enzymes as interchangeable "parts" that can be freely imported into the host organism of choice irrespective of origin. We recognize that these enzyme parts may need to be re-engineered for optimal activity in the target host.
- We believe that advances in protein engineering permits the specification of pathways by considering primarily the functional specificity of an enzyme and assuming that this enzyme may need to be altered to accept new substrates.

We proposed biosynthetic pathways towards glucaric acid by searching the available databases for both specific conversions (e.g., the production of glucaric acid from glucuronic acid by *Pseudomonas syringae* uronate dehydrogenase) and generalized

enzyme functions (e.g., the conversion of an aldehyde to a carboxylic acid through EC class 1.2.1 enzymes). Our proposed pathways are illustrated in Figure 1. We do not attempt an exhaustive search of all theoretical conversions, but rather focus on those most likely realizable based on the prevalence of enzymes within a certain reaction class. Given a pathway design, we attempt its construction by recruiting enzyme activities through PCR amplification of known genes, chemical synthesis of codon-optimized DNA fragments for expression in *E. coli*, and cloning of un-sequenced genes using genomic DNA libraries and/or protein purification. As stated previously, successful construction of some pathways will require enzyme engineering to achieve desired conversion steps.

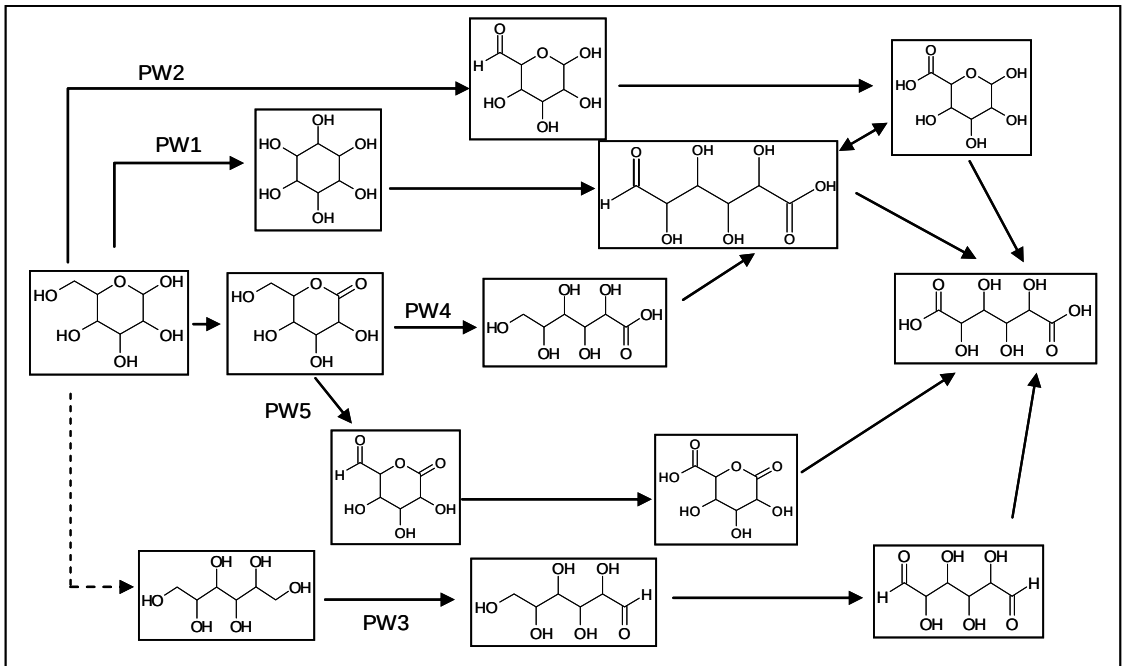

Figure 1. Proposed biosynthetic routes from glucose or sorbitol to glucaric acid. The dashed arrow represents the conversion of glucose to sorbitol. PW1 is the benchmark pathway. Chirality of structures omitted for visual clarity.

We have identified one pathway that utilizes only naturally-occurring enzymes but from disparate sources (Figure 1, PW1). A second designed pathway has been selected that requires enzyme engineering for two of three reaction steps (Figure 1, PW2). We have established collaborators in protein design to achieve this goal.

III. Accomplishments

Our goals begin with specifying pathways for production of the target compound of interest. We first conducted experiments designed to validate our choice of target compound to explore principles of retro-biosynthesis, then proceeded with pathway specification. The methodologies used to propose 5 pathways and select two for assembly have already been described in Section II and will be elaborated upon here. Following pathway specification, we aim to assemble two different routes for microbial production of glucaric acid. The first pathway, PW1, requires an enzyme that prior to the start of this project, had not been cloned. Hence, assembly of this pathway required us to

first identify the gene coding for the enzyme. The second pathway, PW2, requires engineered enzymes and thus required us to establish research collaborations to accomplish this work.

A. Evaluation of glucaric acid toxicity and metabolism in *E. coli*

To successfully construct a heterologous pathway for unnatural product formation, it is best if the target compound has limited effect on host growth. We evaluated the growth of *E. coli* strain DH10B in both LB (complex) and M9/glucose minimal media with varying concentrations of glucaric acid. In all cases, the media was adjusted to pH 7.4 following the addition of glucaric acid. The results in LB indicated that growth rates were unaffected by glucaric acid, while maximum cell densities increased with increasing glucaric acid. This is consistent with reports that *E. coli* can metabolize glucaric acid, but also demonstrates that it provides no growth advantage in rich medium. Minimal medium was used to evaluate the effect of glucaric acid on both growth and glucose uptake rates. Glucaric acid concentrations up to 50 mM (~10 g/L) produced no effect on growth or glucose uptake rate. Maximum cell densities also appeared to be independent of glucaric acid, suggesting catabolite repression. We subsequently confirmed that neither the DH10B nor BL21(DE3) strains of *E. coli* will metabolize glucaric acid in the presence of glucose. This fact allowed us to test the production of glucaric acid in these strains without the need to mutate the host to prevent glucaric acid consumption. Using excess glucose to promote catabolite repression of glucaric acid-utilizing enzymes enabled a rapid determination of pathway success.

B. Specification and selection of biosynthetic pathways

Various routes were proposed to glucaric acid by considering functional group transformations that could achieve the target product and working backwards to arrive at a starting substrate that would be readily taken up by or produced within the cell (Figure 1). One pathway, PW1, was developed by restricting the database search to known conversions only. Since glucaric acid is an oxidized form of glucose, it is reasonable to expect that glucose can serve as such a substrate. Four of the five pathways do originate from glucose. Pathway 3 originates from sorbitol; however, sorbitol can be produced enzymatically from glucose. Therefore, all five pathways could originate from glucose. In selecting conversion steps, we attempted to restrict ourselves to enzyme transformations that were most common, as reflected in a substantial number of enzymes within a particular 3-digit EC group. We believe that common transformations are more likely to be successfully engineered for new substrates. In this manner, we excluded reactions such as the direct oxygenation of glucose, in favor of the two-step oxidation of the primary alcohol of glucose to an aldehyde, followed by oxidation of the aldehyde to a carboxylic acid (Figure 2, PW2). Note that three of the five pathways proceed through glucuronic acid (in either of two equilibrium forms) as the penultimate compound, suggesting an important role of the glucuronic acid to glucaric acid converting enzyme, uronate dehydrogenase.

As stated previously, Pathway 1 was assembled by restricting the database search to known bioconversions. That is, there is documentation in the print literature and online databases that establishes (1) the production of glucaric acid from glucuronic acid by *Pseudomonas syringae* uronate dehydrogenase; (2) the conversion of myo-inositol to glucuronic acid with the mammalian myo-inositol oxygenase (MIOX); and (3) the production of myo-inositol from glucose by inositol 3-phosphate synthase from *S. cerevisiae*. Because each of these reactions was known, PW1 was designated the benchmark pathway. Our objective is to propose an alternative pathway with productivity above the benchmark pathway. Pathway 2 was chosen as the test pathway because it has only 3 steps, utilizes one enzyme of the benchmark pathway, and its first two steps are, generally speaking, common enzymatic transformations.

C. Cloning uronate dehydrogenase activity from *Pseudomonas syringae*

Three of the five proposed pathways, including both of our selected pathways for implementation (PW1 and PW2), rely upon the activity of an enzyme that has been described in *P. syringae* but for which the corresponding gene had not been identified. This enzyme is uronate dehydrogenase, EC 1.1.1.203, and it catalyzes the final conversion step of glucuronic acid to glucaric acid. Thus, we first verified the existence of the desired activity in the native organism. The conversion of glucuronic acid to glucaric acid by *Pseudomonas syringae* grown on either of these compounds but not on glucose has been reported. However, the published report demonstrated the conversion through monitoring of NADH production. To verify this activity, we grew *P. syringae* in both gluose and glucaric acid, prepared cell-free lysates, and tested for glucuronic acid-converting activity. As expected, NADH co-factor was produced in the presence of glucuronic acid and when cells were grown on glucaric acid, but was not produced in the absence of the substrate or when cells were grown on glucose. We have also developed methods for the separation of glucuronic acid and glucaric acid followed by HPLC analysis. The separation properties and HPLC retention times confirmed that *P. syringae* contains glucaric acid-producing activity.

In attempting to establish a simple growth-coupled screen to isolate uronate dehydrogenase, we observed a growth difference in *E. coli* strain DH10B on glucaric and glucuronic acid which we hoped to exploit as the basis for such a screen. This method encountered difficulties when we observed that plasmid-transformed cells exhibited markedly different growth behavior compared to plasmid-free cells. As an alternative, we reviewed the metabolic pathways for growth of *E. coli* on both glucuronic and glucaric acids and determined that catabolism proceeded through two unrelated pathways. Thus, by intentionally disrupting the first step for glucuronic acid consumption (*uxa*C) while retaining the route for glucaric acid consumption, we could screen for uronate dehydrogenase activity by growth of an *E. coli* library transformed with *P. putida* genomic DNA on minimal medium containing only glucuronic acid as a carbon source. Resulting cells might then contain uronate dehydrogenase, which would produce and consume glucaric acid for growth (Figure 2). Using a *uxa*C mutant harboring a *P. syringae* genomic DNA library, we successfully identified open reading frame PSPTO_1053 as coding for uronate dehydrogenase activity (deposited as GenBank

Accession Number EU377538). From this sequence, homologues were identified and tested in *P. putida* and *Agrobacterium tumefaciens* (Figure 3). A manuscript describing this cloning of uronate dehydrogenase from these three organisms has been published (Yoon et al., 2009).

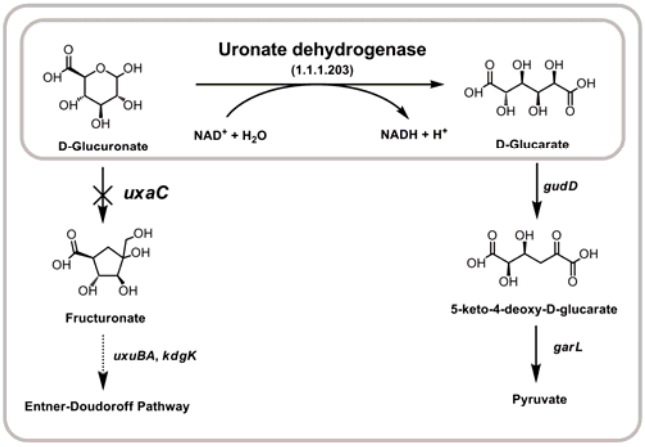

Figure 2. Catabolism of glucuronic and glucaric acids in *E. coli*

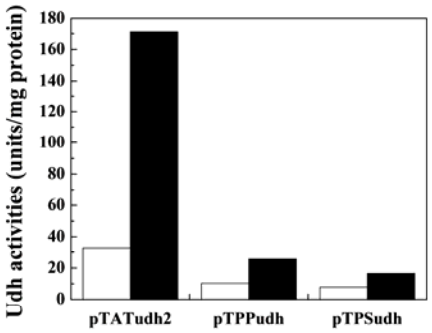

Figure 3. Uronate dehydrogenase activities of clones harboring *udh* gene of *A. tumefaciens* str. C58 (pTATudh2), *P. putida* KT2440 (pTPPudh), and *P. syringae* pv. tomato str. DC3000 (pTPSudh). Open and solid bars represent cultures grown without or with induction by 0.1 mM IPTG, respectively.

While cloning the *udh* gene as described above, we also pursued secondary, bioinformatics-based approaches towards cloning this gene. Based solely on the chemistry, enzymes of the class 1.2.1.x should be capable of converting glucuronic acid to glucaric acid. The genome of *P. syringae* is sequenced, and there are 8 ORFs with putative 1.2.1 activity. We cloned each of these 8 genes and tested them for uronate dehydrogenase activity; however, all 8 failed to display the desired activity. The *udh* that we did identify was not consistent with these open reading frames. More recently, a "uronate dehydrogenase" sequence from grape was deposited in GenBank (Accession DQ843600). We obtained a synthesized version of this gene and it also did not display the desired activity. The latter gene displayed very little (10%) identity with our uronate dehydrogenases. Although difficulties in expression of a grape gene in *E. coli* cannot be ruled out, the low homology leads us to believe that this gene is either not in fact a uronate dehydrogenase, or it is a highly divergent version. This experience emphasizes

the need for additional experimental results to guide bioinformatic efforts to assign enzyme function from sequence data alone.

D. Assembly of the benchmark pathway

The benchmark pathway consists of myo-inositol-1-phosphate synthase, encoded by the INO1 gene, from yeast (*Saccharomyces cerevisiae*); myo-inositol oxygenase, MIOX, from mouse; and uronate dehydrogenase, Udh, from the bacterium *Pseudomonas syringae*, to produce glucaric acid from glucose. The INO1 gene was amplified through PCR amplification from the yeast genome and synthesized. The MIOX gene was synthesized by DNA with codon usage optimized for expression in *E. coli*. The mouse variant of this enzyme was chosen because of previous reports of its functional expression in *E. coli* (Arner et al. 2004. Biochem. Biophys. Res. Commun. 324:1386–1392). Similarly, INO1 expression had been shown to lead to high levels of *myo*-inositol accumulation in recombinant *E. coli* cultures (Niu et al. 2003. J.A.C.S. 125:12998–12999). To establish the benchmark pathway, we first set out to produce glucuronic acid through the expression of recombinant INO1 and MIOX genes.

Expression studies revealed that both enzymes needed high gene dosage levels to result in accumulation of significant amounts of their respective products (*myo*-inositol and glucuronic acid in the culture medium). We subsequently co-expressed the two genes, each under the control of a separate T7 promoter, and achieved production of glucuronic acid from glucose at ~0.3 g/L (Figure 4). The resulting profiles indicated that MIOX activity was rate-limiting, as evidenced by an accumulation of myo-inositol and low activity of the MIOX enzyme (data not shown).

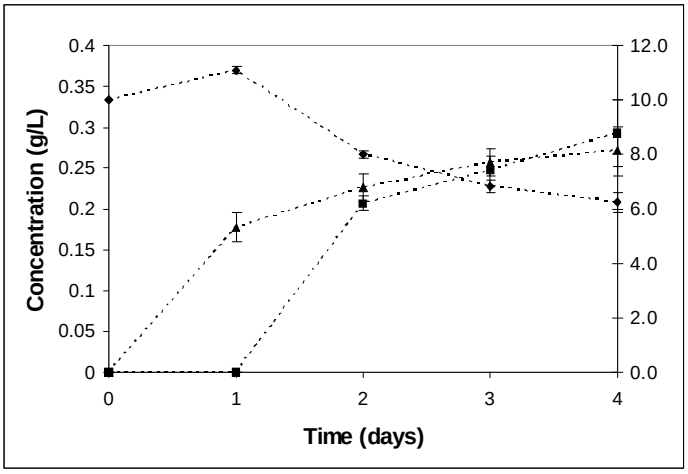

Figure 4. Production of glucuronic acid from glucose in *E. coli*. Cultures were grown in triplicate at 30°C in LB medium supplemented with 10 g/L glucose and 0.1 mM IPTG. Data points are the average and standard deviation of the three biological replicates. ▲ = Glucuronic acid; ■ = *myo*-inositol; ♦ = Glucose.

The cloned *udh* from *P. syringae* was next co-expressed with the first two enzymes of the pathway, leading to production of glucaric acid from glucose. Although the highest recombinant Udh activities were observed with the cloned gene from *A. tumefaciens*, the

activity of Udh from *P. syringae* was two orders of magnitude higher than INO1 and three orders of magnitude higher than MIOX (data not shown). Thus, it was sufficient to observe glucaric acid production (Table 1). Interestingly, significantly more glucaric acid was produced in the three enzyme system than glucuronic acid produced from the two enzyme system. We believe that the high activity of Udh effectively pulls flux through the system, resulting in much higher glucaric acid concentrations. Manipulation of inducer concentrations produced a titer of ~1 g/L. ***This is the first demonstration of microbial production of glucaric acid, a "top value-added" product that can be produced from biomass.*** A manuscript describing the establishment of this pathway has been published (Moon et al., 2009a).

Table 1. Production of glucaric acid from glucose after 3 days culture. Cultures were grown at 30°C in LB medium supplemented with 10 g/L glucose and induced with IPTG. OD_{600} = optical density at 600 nm, Yield (%) = 100 x glucaric acid produced / glucose consumed (mol/mol). Condition A = 0.1 mM IPTG at 0 hr; Condition B = 0.05 mM IPTG at 0 hr; Condition C = 0.05 mM IPTG at 0 hr and 0.1 mM IPTG at 17.5 hr. N/D = not detectable.

Condition	OD_{600}	Glucose (g/L)	*myo*-Inositol (g/L)	Glucuronic Acid (g/L)	Glucaric Acid (g/L)	Yield (%)
A	5.0	6.5	0.09	N/D	0.82	20.0
B	6.3	1.8	0.13	N/D	1.13	11.9
C	5.6	3.6	0.17	N/D	0.88	11.8

E. Improvement of productivity through co-localization of enzymes

The accumulation of myo-inositol in the culture medium, combined with undetectable levels of glucuronic acid indicated that MIOX activity was rate-limiting in the system. Additional studies also showed that MIOX activity was strongly dependent on the presence of *myo*-inositol in the system, an observation that had been previously reported (Arner et al., 2004). In an attempt to increase the titer, we formed a collaboration with Dr. John Dueber at the University of California, Berkeley, to utilize synthetic scaffolds to co-localize the INO1 and MIOX proteins. The INO1 and MIOX proteins were tagged with ligands, and a special scaffold was produced with the ligand-binding peptides in different stiochiometries. This results in INO1 and MIOX molecules being co-localized within the cytoplasm of the cell. Our hypothesis was that co-localization might increase the local concentration of *myo*-inositol (by preventing dilution by diffusion), thereby impacting the MIOX activity. Utilizing the scaffolds resulted in improvements in glucaric acid titers up to nearly 2 g/L. ***The use of synthetic biology parts facilitates the improvement of the system, towards titers necessary to validate biological synthesis as a suitable route for glucaric acid production.*** This work has since been published in *Nature Biotechnology* (Dueber et al., 2009).

E. Collaboration for protein design

In addition to the benchmark pathway utilizing naturally-occurring enzymes, we have proposed a designed pathway based only on generalized enzyme transformations (Figure

1, PW2). This alternative pathway requires two engineered enzymes, and we established a collaboration with Codon Devices (Cambridge, MA) to re-design the enzyme catalyzing the first transformation step. This reaction is the oxidation of glucose to form glucodialdose. A galactose oxidase is known to catalyze the desired reaction using galactose as a substrate. Hence, the objective of the protein engineering project is to use computational methods to re-design the enzyme for activity on glucose, to achieve higher activities than what has previously been reported. We received the first library, a control library, to screen for a new glucose oxidase activity from Codon Devices towards the end of the reporting period.

F. Modeling designed pathways

We have also collaborated with Prof. Alfonso Jaramillo at École Polytechnique (France) on the development of a mathematical model to predict the metabolic burden imposed by the expression of heterologous pathways. The model is designed to both estimate the demand required to transcribe and translate plasmid-encoded genes, and to consider the impact of a heterologous pathway on growth rate through the use of a stoichiometric flux-balance model. ***These types of models will be useful as a metric to choose from among many different options that will necessarily result from designed biosynthetic pathways.*** This work has been published in *Bioinformatics* (Rodrigo et al., 2008).

G. Development of novel experimental devices for controlling glucose flux

We have designed a strategy to control flux of glucose between endogenous metabolism and synthetic pathways based on altering transport (Figure 5). Glucose normally enters the cell through the phosphotransferase system (PTS), and is converted to glucose-6-phosphate before entry into the cytoplasm. It can then enter glycolysis or the pentose phosphate pathway. An alternative transport system can be developed by eliminating the PTS and instead using the galactose permease transporter. Glucose that enters through the permease is phosphorylated by glucokinase after entry into the cytoplasm. We have therefore designed a "metabolite valve" to modulate glucokinase activity in order to control the distribution of glucose that enters endogenous metabolism versus our test pathway for glucaric acid synthesis.

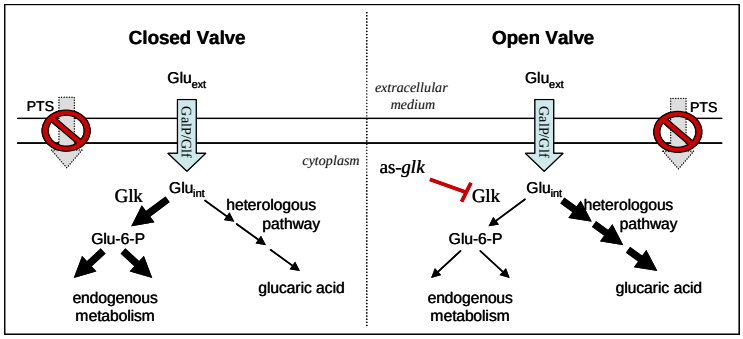

Figure 5. Design of a Glucose "Metabolite Valve." Altering the glucose transport system allows the molecule to enter in a non-phosphorylated form. Controlling phosphorylation should control the entry of glucose into endogenous metabolism. PTS = PEP-dependent phosphotransferase, GalP = galactose permease, Glf = glucose facilitator protein, Glk = glucokinase. Glu = glucose (ext = extracellular, int = intracellular), Glu-6-P = glucose-6-phosphate. as-*glk* = antisense RNA directed towards glucokinase mRNA.

We constructed several strains with mutations in components of the PTS as well as with overexpression of the galactose permease (*gal*P) and glucokinase (*glk*) to achieve maximal growth in the absence of an engineered pathway. Antisense transcripts intended to modulate Glk activity were also designed. While this project was conceived during the period and with the help of ONR funding, follow-up studies and demonstration of the feasibility of this approach were completed through leveraged funding (National Science Foundation).

H. Development of a database tool for retro-biosynthetic pathway design.

In the process of proposing pathways for glucaric acid and other compounds in our lab, we found the current enzyme and metabolism databases to be sub-optimal for the selection of enzymes according to generalized enzyme functions. For example, we were unable to search for "aldehydes" as a functional group and obtain a list of the generic substrates from which an aldehyde could be produced, connected to the appropriate enzyme activity. To address this problem, we developed a database called ReBiT (*Re*tro-*Bi*osynthesis *T*ool) that catalogs enzyme activities as defined by the first 3 digits of the EC number together with only the functional groups that are converted/produced in the substrate/product pair. Using the database, one can, for example, identify all enzymatic reactions that lead to the formation of a primary amine as a functional group. ReBiT contains 605 structures involved in 637 enzymatic reactions. The database can be searched by drawing structures, entering SMILES notation, or browsing a list of functional group names. One can also browse images of all functional groups. The database has been made publicly available through a web interface, **http://www.retro-biosynthesis.com**. ReBiT was also described in a manuscript (Martin and Prather, 2008).

IV. Conclusions

Our goal in establishing this project was to develop methodologies and tools to aid in the design and assembly of novel biosynthetic pathways. To both *investigate* the challenges inherent in this approach and *demonstrate* the feasibility of novel pathway design, we chose to design and assembly routes towards glucaric acid, a compound of increasing commercial interest. Through this work, we successfully achieved microbial synthesis of this compound for the first time. We also encountered difficulties, including the lack of enzyme databases to facilitate chemistry-focused searches, a lack of appropriate bioinformatic tools to identify needed enzymes, and poor activity of mammalian enzymes in a bacterial host. In confronting these difficulties, however, we also developed new tools, both computational/database and experimental in nature. These include mathematical models to help prioritize designed pathways and novel protein scaffolds to boost activity of key enzymes (both accomplished through collaborations). In summary, we achieved our initial goal of microbial synthesis of glucaric acid and demonstrated the promise of novel pathway design for biological production of organic compounds.

V. Significance

The significance of this work can be assessed from two perspectives. First, our ability to *de novo* design and assemble pathways towards synthesis of an organic compound serves as a proof-of-concept of the ability to branch out from known biological routes towards the synthesis of desired targets in the absence of a pre-defined route. As we seek to uncover sustainable sources of fuels and chemicals, the tools we have developed as well as our experiences in establishing the benchmark pathway should facilitate future pathway design/assembly efforts. Second, we chose as our target compound glucaric acid, a molecule that has been declared as a "top value-added chemical" and which is often characterized as a molecule with much potential save for the current expense of manufacturing. Thus, our work could potentially lead to an alternative, sustainable, and more economical means of producing this high-value compound.

VII. Publications

a. Refereed Journal Articles

The following manuscripts were submitted for review during the award period:

1. Prather, K.L.J. and Martin, C.H. 2008. "*De novo* biosynthetic pathways: rational design of microbial chemical factories." *Curr. Opin. Biotechnol.* **19(5)**:468-474.

2. Rodrigo, G., Carrera, J., Prather, K.L.J., and Jaramillo, A. 2008. "DESHARKY: Automated design of metabolic pathways for optimal cell growth." *Bioinformatics.* **24(21)**:2554-2556.

3. Moon, T.S., Yoon S.-H., Lanza, A.M., Roy-Mayhew, J.D. and Prather, K.L.J. 2009a. "Production of glucaric acid from a synthetic pathway in recombinant *Escherichia coli*." *Appl. Environ. Microbiol.* **75(3)**:589-595.

4. Yoon, S.-H., Moon, T.S., Iranpour, P., Lanza, A.M., and Prather, K.J. 2009. "Cloning and characterization of uronate dehydrogenases from two Pseudomonads and *Agrobacterium tumefaciens* str. C58." *J. Bacteriol.* **191(5)**:1565-1573.

The following manuscripts were submitted for review after the award period; however, the work described was performed during the reporting period and with ONR support:

1. Moon, T.S., Yoon S.-H., Tsang Mui Ching, M-J., Lanza, A.M.. and Prather, K.L.J. 2009b. "Enzymatic assay of D-glucuronic acid using uronate dehydrogenase." *Analytical Biochemistry*. **39**:183-185.

2. Dueber, J.E., Wu, G.C., Malmirchegini, G.R., Moon, T.S., Petzold, C.J., Ullal, A.V., Prather, K.L.J., Keasling, J.D. 2009. "Synthetic protein scaffolds provide modular control over metabolic flux." *Nature Biotechnol.* **27(8)**:753-759.

b. Non-Refereed Significant Publications

none

c. Books or Chapters

none

d. Workshop/Conference abstracts, presentations, posters, or papers

1. *Metabolic Engineering VI, The Netherlands, October 2006.* Poster: "Novel Pathway Design for Microbial Production of Organic Compounds." Kristala Jones Prather [Presenter], Collin Martin, Pooya Iranpour, Tae Seok Moon

2. *AIChE Annual Meeting, San Francisco, CA, November 2006.* Poster: "Development of a database tool for novel biosynthetic pathway design." Wei Chan, Amanda Lanza, Kristala Jones Prather [Presenter]

3. *Biochemical Engineering XV, Quebec City, QC, Canada, July 2007*. Poster: "Towards microbial synthesis of glucaric acid." Tae Seok Moon, Pooya Iranpour, Amanda Lanza, Leah Octavio, Kristala Jones Prather [Presenter]

4. *American Chemical Society Fall Annual Meeting, Boston, MA, August 2007*. Paper: "Towards microbial synthesis of glucaric acid." Tae Seok Moon [Presenter], Pooya Iranpour, Amanda Lanza, Leah Octavio, Kristala Jones Prather

5. *American Chemical Society Fall Annual Meeting, Boston, MA, August 2007*. Poster: "ReBit: a database for enzymatic pathway design." Collin Martin [Presenter] and Kristala Jones Prather

6. *AIChE Annual Meeting, Salt Lake City, UT, November 2007*. Paper: "Towards microbial synthesis of glucaric acid." Tae Seok Moon [Presenter], Sang-Hwal Yoon, Leah Octavio, Kristala Jones Prather

VIII. Patent Information

"Cellular production of glucaric acid," with Tae Seok Moon and Sang-Hwal Yoon. PCT Patent Application (PCT/US2009/002111) filed April 3, 2009

IX. Technology Transfer

- **Leveraging of ONR funding** – The fundamental principles of retro-biosynthesis first described in the ONR proposal have subsequently been integrated into a successfully funded proposal for an NSF-sponsored Engineering Research in

Synthetic Biology (SynBERC). Since the end of ONR support on 31May2008, this project has been supported by SynBERC.

- **Future plans for technology transfer** – Glucaric acid has been identified as a "top-valued added" product from biomass. We anticipate that continued improvements in production of this compound through biological means will generate interest from biomass companies.

X. Awards/Honors

2006 – Outstanding Faculty Award for Undergraduate Teaching, Dept of Chemical Engineering (MIT)
2007 – Technology Review "TR35" Young Innovator